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BASIC FIBROBLAST GROWTH FACTOR IN RAT CORPUS LUTEUM STIMULATES PROSTAGLANDIN F2-ALPHA PRODUCTION

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SUMMARY: Rat luteal cells (LC) were incubated with or without basic fibroblast growth factor (bFGF) in a serum-free medium. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production was stimulated in a dose-dependent manner at 0.03 ~ 1 ng/ml of bFGF. One ng/ml of bFGF caused approximately 5-fold the increment of $PGF_{2\alpha}$ at every stage of LC after 48 hrs of incubation. bFGF also raised progesterone secretion from LC, and this stimulatory effect on progesterone was more distinguishable in an early-, than a middle- and a late-stage. Additionally, bFGF concentration throughout the luteal phase was assessed using western blot analysis. The protein with typical molecular weight 18 kDa form was in high concentration throughout the luteal phase. These results suggest that bFGF may play a role in the regulation of $PGF_{2\alpha}$ and progesterone production as autocrine, but not in mitosis in corpus luteum.

Recently, growth factors synthesized in the ovary have been reported to have possibilities to regulate ovarian steroidogenesis and modulate the gonadotropin actions onto ovarian function (1,2). Basic fibroblast growth factor (bFGF) which was originally identified in the pituitary gland and brain, was also isolated from bovine corpus luteum (CL) (3). Moreover, the mRNA encoding bFGF was expressed in both luteal cells (4) and granulosa cells (5). These facts prompted us to study the biological role of bFGF in luteal function. In addition, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is well known as one of the important factors for regulation of luteal function (6,7,8) and it has been shown that $PGF_{2\alpha}$ of luteal origin as well as that of uterus-derived may be involved in the

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The abbreviations used are bFGF, basic fibroblast growth factor; $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$; PRG, Progesterone; CL, Corpus luteum; LC, Luteal cells; PMSG, pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin.

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luteolysis in rats (9). Therefore, we studied the effects of bFGF on the $PGF_{2\alpha}$ and progesterone synthesis by luteal cells in vitro.

MATERIALS AND METHODS

Animals and preparation of CL: In order to obtain a well-defined generation of CL, immature female rats of the Sprague-Dawley strain (24~32-day-old) were sc injected at 0800 with 8 IU of pregnant mare serum gonadotropin (PMSG), followed by an ip injection with 10 IU human chorionic gonadotropin (hCG) at 1700 2 days after PMSG injection (normal ovulation model). Ovulation took place in the morning of the next day after hCG injection (10) and an average of 13 ± 3.2 (n=9) oocytes were observed in all rats. The day of hCG administration was defined as day-0 and the CL isolated from the rat which was observed ovulating was designated as a 1-day-old CL, with consecutive numbering up to a 9-day-old CL (9 days after hCG treatment). For the experiment of western blotting, higher doses of 50 IU PMSG and 25 IU hCG were used to obtain a sufficient number of luteal cells. However, it is known that no significant differences were found between the two models with regard to the microscopic appearance of LC or the response to various stimuli (11).

Luteal cell preparation : All CL removed from ovary was cleaned off adhering ovarian tissue under a dissecting microscope. The CL tissue was digested in Ca^{2+}- and Mg^{2+}- free phosphate buffer saline containing 0.2 % collagenase, 250 µg/ml DNase and 1 % fetal bovine serum in a shaking water bath at 37 °C for 90 min. After cell suspension was filtered through a nylon mesh, luteal cells (LC) were collected by centrifugation and then washed 3 times in an incubation medium.

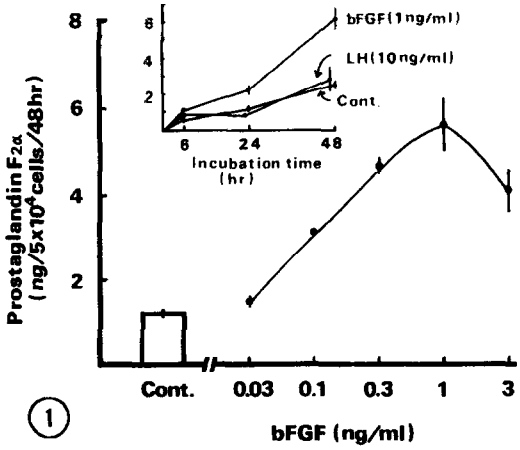
Cell culture and $PGF_{2\alpha}$ and progesterone assay: LC was dispersed in serum-free culture medium comprised of 1:1 mixture of Eagle's minimum essential medium and Ham'F 12 supplemented with 10 mM Hepes, 2 mM L-glutamine and antibiotics (100 U/ml penicillin, 100 ug/ml streptomycin and 625 ng/ml fungizone). Cells were incubated at a density of 5×10^4 cells/dish in 500 µl medium at 37 °C with or without recombinant bFGF (human; Takeda) or LH (ovine; NIADDK-oLH-26) under a water saturated atmosphere of 5 % CO_2 and 95 % air. The amount of $PGF_{2\alpha}$ was determined using RIA kit (Baxter Healthcare Corp.). The content of progesterone (PRG) was measured by RIA with specific antiserum (BioMaker, Israel) as described in detail earlier (12,13). The coefficients of the intra- and inter-assay variations in each RIA were smaller than 10 %. Experimental data are presented as the mean ± SE and evaluated by analysis of variance with Bartlett's test followed by Student's t-test. A difference of $P<0.05$ was considered significant.

Western blot analysis: CL tissues (200 mg) of each day-old (3,6 and 9) were homogenized with 2 ml of 25 mM tris-HCl buffer (pH 7.45) containing 3 M NaCl, 1 mM phenylmethylsulfonyl fluoride , 4 µM leupeptin and 2 µM pepstatin at 4 °C using a glass homogenizer. After 10 min., the homogenates were diluted to 0.5 M NaCl with 25 mM tris-HCl containing protease inhibitors and centrifuged twice at 14,000 x g for 15 min at 4 °C. Supernatants were loaded onto 50 µl of heparin-sepharose column. The column was washed with 25 mM tris-HCl containing 50 ml of 1 M NaCl and then resin particles were transferred to an Eppendorf tube and boiled for 3 min in SDS-PAGE sample buffer. Buffer containing recombinant bFGF was also

performed the same procedure with the column. Proteins were electrophoresed on SDS-15 % polyacrylamide gel and were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon). The membrane was incubated in 25 mM tris-saline buffer containing 1 % BSA at room temperature with Iodinated [^{125}I]-anti-bFGF monoclonal antibody (5 x 10^5 cpm/ml, MoAb 98, Takeda) for 5 hr, and then washed and exposed to film (Kodak X-OMAT AR X-ray film) for an autoradiography (-70 °C).

RESULTS AND DISCUSSION

$PGF_{2\alpha}$ production under various concentrations (0.03-3 ng/ml) of bFGF in 3-day-old LC was measured (Fig. 1). As shown in fig. 1, bFGF stimulated the $PGF_{2\alpha}$ production between 0.03 and 1 ng/ml of bFGF in a dose-dependent manner. The $PGF_{2\alpha}$ levels significantly increased by treatment with 0.1 ng/ml of bFGF ($P<0.01$; as compared with control) and the maximum response was observed at 1 ng/ml of bFGF. Time-course studies (the inset in Fig. 1) revealed that bFGF (1 ng/ml) significantly stimulated the $PGF_{2\alpha}$ production by 6 hrs



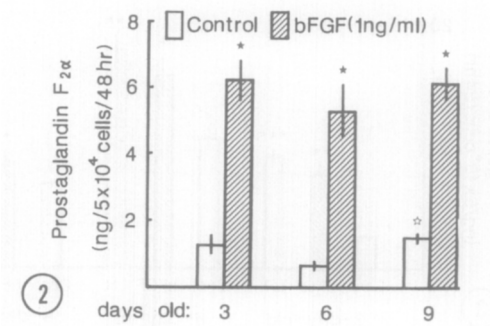
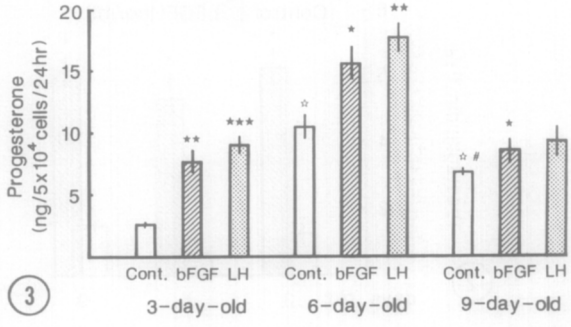


Fig. 1. Dose response effect of bFGF on $PGF_{2\alpha}$ production in cultured rat luteal cells.
Immature rats were injected with hCG (10 IU, ip) at 57 hrs after PMSG (5 IU, sc) treatment. Luteal cells, which were collected 3 days after hCG treatment, were cultured for 48 hrs in the presence of various doses of bFGF. $PGF_{2\alpha}$ in the medium was determined by RIA. Each value shows the mean ± SE of quadruplicate cultures. Similar results were obtained by three additional experiments. The inset shows the time course of the effect of bFGF on $PGF_{2\alpha}$ production. A maximal stimulatory dose of bFGF (1 ng/ml) was used. Each point represents the mean ± SE of triplicate cultures.

Fig. 2. Stimulatory effects of bFGF on $PGF_{2\alpha}$ production in luteal cells 3, 6 and 9 days after hCG treatment.
Luteal cells, which were collected 3, 6 and 9 days after hCG treatment, were cultured for 48 hrs in the presence of bFGF (1 ng/ml). $PGF_{2\alpha}$ in the medium was measured by RIA. Each value shows the mean ± SE of 12 cultures obtained in a third series of identical experiments. ★$P<0.001$: vs. control of each day old. ☆$P<0.01$: vs. control of 6-day-old.

of culture, and afterwards the $PGF_{2\alpha}$ levels elevated gradually during the 48 hrs culture period. Treatment for 48 hrs with bFGF had no effect on cell number, suggesting that the stimulative effect of bFGF can not be attributed to an increase of cell number. But, the $PGF_{2\alpha}$ levels in the LH (10 ng/ml)-treated group were similar to that of the control group during the 48 hrs, although the PRG levels in this group were stimulated at 24 hrs of culture as shown in Fig. 3. We also observed that bFGF in combination with LH tended to inhibit the ability of bFGF to stimulate the luteal $PGF_{2\alpha}$ production (data not shown). The stimulatory effect of bFGF on the $PGF_{2\alpha}$ production was also recognized on 6- or 9-day-old LC as observed on 3-day-old LC (Fig. 2). Levels of bFGF-stimulated $PGF_{2\alpha}$ were almost the same in each day-old, although control levels on 9-day-old LC significantly increased as compared with that of a 6-day-old. Next, the effect of bFGF (1 ng/ml) on luteal PRG production was studied on 3-, 6- and 9-day-old LC (Fig. 3). PRG levels were measured at 24 hrs after the culture started, because the PRG levels in control or LH-treated group peaked between 12 and 24 hrs of culture as previously re-



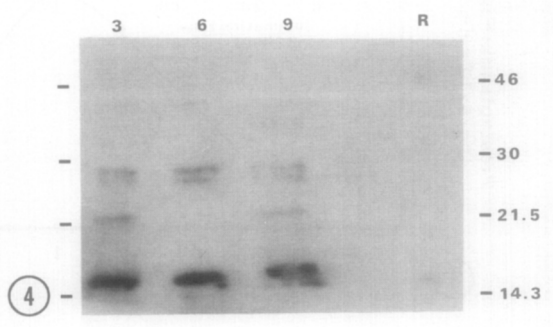


Fig. 3. Effects of bFGF on progesterone production in luteal cells 3, 6 and 9 days after hCG treatment.
Luteal cells, which were collected 3, 6 and 9 days after hCG treatment, were cultured for 24 hrs in the presence of bFGF (1 ng/ml) or LH (10 ng/ml). The progesterone level in the medium was measured by RIA. Each value shows the mean ± SE of quintuplicate cultures. Similar results were obtained in five additional experiments. ★P<0.05, ★★P<0.01, ★★★P<0.001 vs. control of each control. ☆P<0.01 vs. control of 3-day-old. #P<0.01 vs. control of 6-day-old.

Fig. 4. Western blot analysis of heparin-affinity purified extracts of luteinized ovary probed with [^{125}I]-bFGF monoclonal antibody.
Immature rats were injected with hCG (25 IU, ip) at 57 hrs after PMSG (50 IU, sc) treatment. Luteinized ovaries were collected 3, 6 and 9 days after hCG treatment. Proteins extracted from their ovaries and recombinant bFGF (5 ng) were analysed by western blot, as described in Materials and Methods. Molecular weight standards are indicated in thousands. 3: 3-day-old, 6: 6-day-old, 9: 9-day-old, R: recombinant bFGF.

ported (13). bFGF raised PRG secretion from LC of every day-old examined, as in the case of $PGF_{2\alpha}$. bFGF which was purified from human placenta also stimulated the PRG release from 3 ~ 4-day-old LC (14). The stimulative effect on PRG production gradually decreased as CL aged, as compared with each control level (2.68-fold in 3-day-old, 1.50-fold in 6-day-old, and 1.24-fold in 9-day-old). We recently observed that bFGF receptor levels decreased as CL aged and that relative high levels of bFGF receptor at day-1 to -4 suddenly fell at the 5-day-old and were almost undetectable thereafter (15). Considering from this result, the decrease of responsibility in PRG production, not in $PGF_{2\alpha}$ production to bFGF may be due to the decrease in the number of receptors for bFGF. But, the levels of bFGF-stimulated $PGF_{2\alpha}$ production was high in spite of a small amount of the receptor in late stage of CL, while PRG production by bFGF addition was parallel with the bFGF receptor site in CL. The reason why constant levels of bFGF-stimulated $PGF_{2\alpha}$ production in all stage of CL is not clear at present. Western blotting analysis using bFGF monoclonal antibody revealed a main band at 18 kDa in heparin affinity purified components which were prepared from 3-, 6- and 9-day-old of luteinized ovaries (Fig. 4). The apparant difference of bFGF levels between each days was not observed. This result indicates that bFGF of high concentration exist in CL thoughout luteal phase. The existence of larger forms of bFGF has been reportedly suggested (16, 17). Figure also showed the presence of immunoreactive bFGF-like proteins in the ovary, which were detected between 21.5 and 30 kDa as reported previously.

However, there is a question whether the bFGF enhances PG and PRG synthesis in LC in vivo. All of the translational products of bFGF mRNA appear to lack a signal sequence which would direct their release via the normal secretory pathway (18,19), whereas bFGF is found in the extracellular matrix (20,21) although the mechanism for its release is not understood. Our result of immunohistochemical staining using the bFGF monoclonal antibody showed that a localization of bFGF was mainly in intracellular compartments of LC (unpublished data). These observations imply that the bFGF molecule may not be fully secreted from the cell and extracellular bFGF concentration in vivo is very low. Therefore, it is difficult to conclude that the doses of bFGF used in this study are physiological concentrations. Assuming that the dose is a physiological concentration in the milieu of LC in vivo, the following discussion will be possible with regard to the physiologi-

cal significance of $PGF_{2\alpha}$ enhanced by bFGF. The exogenous administration of $PGF_{2\alpha}$ has been shown to cause regression of CL (luteolysis) in many species, including the rat (7.8). Olofsson et al. recently reported that $PGF_{2\alpha}$ which was produced locally in the rat ovary may be involved in functional luteolysis (9). In some reports, $PGF_{2\alpha}$ exerts luteolytic action only in old age of CL, whereas the functional CL, namely young age (before day-4 of a pseudopregnant rat), was refractory to $PGF_{2\alpha}$-induced luteolysis (22,23). Moreover, Our observation that bFGF markedly stimulate the PRG production in the early-, than the late-stage of CL may indicate influential role of bFGF in the development and the maintenance of functional CL. Therefore, the possibility will not be denied that $PGF_{2\alpha}$ enhanced by bFGF in old age of CL might have a luteolytic effect. On the other hand, $PGF_{2\alpha}$ has been postuated to have a luteotropic action in CL, because $PGF_{2\alpha}$ can stimulate progesterone production in vitro (24,25). So, to understand the physiological role of bFGF-enhanced $PGF_{2\alpha}$ in LC, further study is required.

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